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RESEARCH ARTICLE

Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate-oxidizing bacteria

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One sentence summary: SAOB were more sensitive to high ammonia compared to the hydrogenotrophic methanogens tested. Thus, hydrogenotrophic methanogens could be equally, if not more, tolerant to high ammonia levels compared to SAOB.
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ABSTRACT

Ammonia-rich substrates can cause inhibition on anaerobic digestion process. Syntrophic acetate-oxidizing bacteria (SAOB) and hydrogenotrophic methanogens are important for the ammonia inhibitory mechanism on anaerobic digestion. The roles and interactions of SAOB and hydrogenotrophic methanogens to ammonia inhibition effect are still unclear. The aim of the current study was to determine the ammonia toxicity levels of various pure strains of SAOB and hydrogenotrophic methanogens. Moreover, ammonia toxicity on the syntrophic-cultivated strains of SAOB and hydrogenotrophic methanogens was tested. Thus, four hydrogenotrophic methanogens (i.e. *Methanoculleus bourgensis, Methanobacterium congolense, Methanoculleu thermophilus* and *Methanothermobacter thermautotrophicus*), two SAOB (i.e. *Tepidanaerobacter acetatoxydans* and *Thermacetogenium phaeum*) and their syntrophic cultivation were assessed under 0.26, 3, 5 and 7 g NH_4^+-N L^{-1}. The results showed that some hydrogenotrophic methanogens were equally, or in some cases, more tolerant to high ammonia levels compared to SAOB. Furthermore, a mesophilic hydrogenotrophic methanogen was more sensitive to ammonia toxicity compared to thermophilic methanogens tested in the study, which is contradicting to the general belief that thermophilic methanogens are more vulnerable to high ammonia loads compared to mesophilic. This unexpected finding underlines the fact that the complete knowledge of ammonia inhibition effect on hydrogenotrophic methanogens is still absent.

Keywords: ammonia inhibition; anaerobic digestion; biogas; SAOB; syntrophic growth

INTRODUCTION

Anaerobic digestion is a biological treatment for organic wastes by which pollution control and renewable energy can be obtained at the same time. Specifically, anaerobic digestion is a multistep process consisting of four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are performed by different groups of microorganisms (Angelidaki *et al.* 2011). However, substrates that contain high total ammonia (NH_4^+ + NH_3) levels can inhibit the anaerobic digestion process and result in suboptimal biogas production (Fotidis *et al.* 2014). The unionized form of ammonia (free ammonia) is considered as the main toxic compound causing ammonia inhibition. Specifically, Sprott and Patel (1986) and Gallert, Bauer and Winter (1998) reported that passive diffusion of free ammonia into the microbes cells is causing proton imbalance, potassium deficiency, increase maintenance energy requirements and suppress specific enzyme reactions. Total ammonia concentration, temperature and pH affect free ammonia concentration in anaerobic digestion process (Chen, Cheng and Creamer 2008). Specifically, the shift from NH_4^+ to NH_3 is enhanced alongside the increase of pH and temperature and results in increased toxicity on the

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anaerobic digestion process (Angelidaki and Ahring 1994). Many investigations have been conducted assessing the inhibition levels of ammonia and/or free ammonia on anaerobic digestion process. In Sung and Liu (2003) study, total ammonia concentrations of 4.92 g NH_4^+-N L^{-1} (55°C, pH 6.71) caused 39% reduction in specific methanogenic activity in continuous stirred tank reactors (CSTR). The results of batch experiments in the same study indicated that ammonia concentrations above 4.0 g NH_4^+-N L^{-1} (55°C, pH from 6.5 to 8.0) had an obvious inhibitory effect on methanogenesis. In continuously fed biogas reactors (Angelidaki and Ahring 1994), inhibition occurred at 4 g NH_4^+-N L^{-1} (free ammonia: 650 mg NH_3-N L^{-1}, 55°C, pH 7.7). In batch experiments conducted in the same study, inhibition was detected at 2 g NH_4^+-N L^{-1} (free ammonia: 140 mg NH_3-N L^{-1}, 55°C, pH 7.9–8.0). Generally, inhibition in continuous reactor experiments will first be detected when the reduced growth rates do not permit the microorganisms to stay in the reactor at specific hydraulic retention times. On contrary, at batch cultivations, the reduction of the growth rates or methane production rates will be identified already at the initial inhibition levels (Liu, Zeng and Angelidaki 2008).

Among all steps of anaerobic digestion process, methanogenesis seems to be the most sensitive to ammonia and thus the rate-limiting step of the anaerobic digestion process (Angelidaki *et al.* 2011). During methanogenesis, acetate is the main precursor to produce methane through two distinct pathways: the aceticlastic pathway and the syntrophic acetate oxidation (SAO) pathway. In aceticlastic pathway, acetate is cleaved to methane and carbon dioxide (reaction 1) by aceticlastic methanogens (i.e. *Methanosaetaceae* spp. and *Methanosarcinaceae* spp.) (Angelidaki *et al.* 2011).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad \Delta G^{0'} = -31.0\ \text{kJ} \cdot \text{mol}^{-1} \tag{1}$$

The SAO pathway can be divided into two descriptive segments: first, SAOB convert acetate into hydrogen and carbon dioxide (reaction 2) and second, hydrogenotrophic methanogens (i.e. *Methanococcales* spp., *Methanobacteriales* spp., *Methanomicrobiales* spp., *Methanocellales* spp. and *Methanopyrales* spp.) use hydrogen and carbon dioxide of the first segment to produce methane (reaction 3) (Zinder and Koch 1984).

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \quad \Delta G^{0'} = +104.6\ \text{kJ} \cdot \text{mol}^{-1} \tag{2}$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \Delta G^{0'} = -135.6\ \text{kJ} \cdot \text{mol}^{-1} \tag{3}$$

The tolerance to ammonia toxicity of the microorganisms involved in the two methanogenic pathways is different (Yenigün and Demirel 2013). Specifically, many studies indicated that hydrogenotrophic methanogens are more robust to ammonia inhibition compared to aceticlastic methanogens (Koster and Lettinga 1984; Angelidaki and Ahring 1993). Although there are many different studies in this field, they often present contradictory results. Specifically, it has been reported that initial inhibition was found at 3.5 g NH_4^+-N L^{-1} and the growth rate was reduced by 50% at 7 g NH_4^+-N L^{-1} (55°C, pH 7.9–8.0) for hydrogenotrophic methanogens (Angelidaki and Ahring 1994). At the same time, other hydrogenotrophic methanogen (*Methanobacterium* strain G2R) did not suffer any ammonia inhibition between 4.2 and 5.6 g NH_4^+-N L^{-1} at 35°C (Sprott and Patel 1986). Therefore, the effect of ammonia on hydrogenotrophic methanogens still needs to be studied.

So far, six SAOB have been isolated; three mesophilic: *Syntrophaceticus schinkii* (Westerholm, Roos and Schnürer 2010), *Tepidanaerobacter acetatoxydans* (Westerholm, Roos and Schnürer 2011) and *Clostridium ultunense* (Schnürer, Schink and Svensson 1996) and three thermophilic: *Thermacetogenium phaeum* (Hattori *et al.* 2000), *Thermotoga lettingae* (Balk, Weijma and Stams 2002) and strain AOR (Lee and Zinder 1988). Specifically, it was reported that pure strains of *C. ultunense*, *T. acetatoxydans* and *S. schinkii* were robust to 8.4–14 g NH_4^+-N L^{-1} (Schnürer, Schink and Svensson 1996; Westerholm, Roos and Schnürer 2010, 2011). In Kato *et al.* (2014)'s study, the results showed that the growth rate of *T. phaeum* decreased when ammonia level increased to 2.8 g NH_4^+-N L^{-1}. However, the tolerance to high ammonia levels (>5 g NH_4^+-N L^{-1}) of *T. lettingae* is still unclear (Sun *et al.* 2014).

The cooperation of SAOB and hydrogenotrophic methanogens is essential in SAO. SAOB do not have the ability to catabolize acetate alone. Hydrogenotrophic methanogens which can use hydrogen and carbon dioxide are necessary to maintain a low partial hydrogen pressure environment so SAOB can keep consuming acetate in SAO pathway (Angelidaki *et al.* 2011). The combination of SAOB and hydrogenotrophic methanogens is based on interspecies hydrogen transfer (Stams *et al.* 2006). Kato *et al.* (2014) reported that the methane production rate of syntrophic coculture (*Methanothermobacter thermautotrophicus* + *T. phaeum*) was barely affected by 1.4 g NH_4^+-N L^{-1} but was reduced by nearly 50% at 2.8 g NH_4^+-N L^{-1} and keep decreasing at 7 g NH_4^+-N L^{-1}. However, so far, information about the effect of different ammonia levels on the syntrophic cultivation of SAOB and hydrogenotrophic methanogens are still lacking. Moreover, the interactions between SAOB and hydrogenotrophic methanogens under different ammonia levels and the significance of each one of them on the performance of the SAO pathway are still unclear. Therefore, the aim of the current study was to assess the effects of different ammonia levels on pure strains of SAOB and hydrogenotrophic methanogens. Moreover, an additional aim was to assess the effect of different ammonia levels on the syntrophic cultivation of SAOB and hydrogenotrophic methanogens.

MATERIALS AND METHODS

Pure strains

Four hydrogenotrophic methanogens (mesophilic: *Methanoculleus bourgensis MS2* DSM No. 3045 and *Methanobacterium congolense C* DSM No. 7095; thermophilic: *Methanoculleus thermophilus* UCLA DSM No. 2624. and *Methanothermobacter thermautotrophicus* Z-245 DSM No. 3720) and two SAOB (Mesophilic: *T. acetatoxydans* $Re1^T$ DSM No. 21804; thermophilic: *T. phaeum strain PB* DSM No. 26808) were purchased from DSMZ company (Germany) and were used for testing their tolerance to ammonia toxicity. Before the main experiments, in order to grow the necessary biomass, all the pure strains were cultivated in the corresponding growth media suggested by the literature (Ollivier *et al.* 1986; Maestrojuan *et al.* 1990; Sonne-Hansen and Ahring 1997; Hattori *et al.* 2000; Cuzin *et al.* 2001; Westerholm, Roos and Schnürer 2011). Specifically, the growth media used were medium 332 for *M. bourgensis*, medium 119 for *M. congolense*, *M. thermophiles* and *M. thermautotrophicus*, medium 1301 for *T. acetatoxydans* and medium 880 for *T. phaeum*.

Table 1. Different total ammonia and free ammonia concentration in pure strains cultivation experiment.

	Experimental steps			
	1st	2nd	3rd	4th
Total ammonia (g NH_4^+-N L^{-1})	0.26	3	5	7
Free ammonia of mesophilic (mg NH_3 L^{-1})	3.31	38.2	63.68	89.15
Free ammonia of thermophilic (mg NH_3 L^{-1})	9.78	112.89	188.14	263.4

Experimental setup

Pure strains cultivation experiments

All hydrogenotrophic methanogens and SAOB were cultivated under four different ammonia and free ammonia concentrations (Table 1) with NH_4Cl as ammonia source. To ensure identical experimental conditions, basal anaerobic media (BA medium) (Angelidaki, Petersen and Ahring 1990) was used for all batch experiments. The BA medium preparation process was as followed: five different stock solutions were prepared for BA medium: (A) NH_4Cl 100 g, NaC1 10 g; $MgCl_2 \bullet 6H_2O$ 10 g, $CaCl_2 \bullet 2H_2O$ 5 g, all chemicals dissolved in distilled water, 1 L total volume. (B) $K_2HPO_4 \bullet 3H_2O$, 200 g dissolved in distilled water, 1 L total volume. (C) Resazurin 0.5 g dissolved in distilled water, 1 L total volume. (D) Trace metal and selenite solution: $FeCl_2 \bullet 4H_2O$ 2 g, H_3BO_3 0.05 g, $ZnCl_2$ 0.05 g, $CuCl_2 \bullet 2H_2O$ 0.038 g, $MnCl_2 \bullet 4H_2O$ 0.05 g, $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ 0.05 g, $AlCl_3$ 0.05 g, $CoCl_2 \bullet 6H_2O$ 0.05 g, $NiCl_2 \bullet 6H_2O$ 0.092 g, ethylenediaminetetraacetate 0.5 g, concentrated HCl 1 mL, $Na_2SeO_3 \bullet 5H_2O$ 0.1 g, all chemicals dissolved in distilled water, 1 L total volume. (E) vitamin mixture (Wolin, Wolin and Wolfe 1963). To 974 mL of redistilled water, the following stock solutions were added: A, 10 mL; B, 2 mL; C, 1 mL; D, 1 mL; E, 1 mL. After boiling with extra water to the original volume, the mixture was cooled under gassing with 80% N_2-20% CO_2. Cysteine hydrochloride (0.5 g) and $NaHCO_3$ (2.6 g) were added and the medium was dispensed and autoclaved. Before inoculation the vials were reduced with $Na_2S \bullet 9H_2O$ to a final concentration of 0.025%.

Batch reactors with 118 mL total and 40 mL working volume, respectively, were used for hydrogenotrophic methanogens cultivation. For SAOB experiments, glass test tubes with 30 mL total volume and 20 mL working volume, respectively, were used. H_2 (62 mL) and CO_2 (16 mL) were added with syringes into closed batch bottles resulting in gas overpressure in the headspace, as substrate for the hydrogenotrophic methanogens. Finally, glucose (1.8 g L^{-1}) and methanol (0.3%) were used as carbon source for *T. acetatoxydans* and *T. phaeum*, respectively, in the SAOB experiments. All the batch reactors and tubes were incubated in their corresponding temperatures (37 ± 1°C for mesophilic and 55 ± 1°C for thermophilic), and all experiments were performed in triplicates (n = 3).

Syntrophic cultivation experiments

Hydrogenotrophic methanogens and SAOB were cultivated together under the same four different ammonia concentrations of the pure strains cultivation experiments. The BA medium was also used in syntrophic cultivation experiments. Batch reactors with 118 mL total and 40 mL working volume, respectively, were used for syntrophic cultivation. Acetate (2 g L^{-1}) was used as carbon source in the experiments. All the batch reactors were incubated in their corresponding temperatures (37 ± 1°C for mesophilic and 55 ± 1°C for thermophilic), and all experiments were performed in triplicates (n = 3).

Analytical methods

Methane accumulation in the headspace of the batch reactors of hydrogenotrophic methanogens and syntrophic cultivation were measured by using Shimadzu-14A gas chromatographer (GC) equipped with a thermal FID detector which uses hydrogen as a carrier gas (Shimadzu, Kyoto, Japan) (Flores *et al.* 2015). For presenting the growth of SAOB, Spectronic 20D+ Spectrophotometer was used for measuring the optical density at 600 nm (OD_{600}) (Thermoscientific, Soeborg, Denmark) (Tomás *et al.* 2013).

Calculations

Free ammonia

The free ammonia concentrations were calculated based on the following equation (Fotidis *et al.* 2013):

$$FAN = \frac{TAN}{1 + \frac{10^{-pH}}{K_a}},$$

where TAN is the total ammonia nitrogen, K_a is a dissociation constant that reflects on temperature with values 1.29×10^{-9} and 3.91×10^{-9} for 37°C and 55°C respectively and pH is equal to the pH of the liquid, which was 7 in the current study.

Growth rate

The maximum specific growth rates (μ_{max}) of hydrogenotrophic methanogens, SAOB and syntrophic cultivation were calculated as the slope of the linear part of the semi-logarithmic graph of the methane production (for SAOB OD_{600} was used) of the batch reactors versus time (Gray *et al.* 2009).

Statistical analysis

The Origin program (OriginLab Corporation, Northampton, Massachusetts) was used for statistical analysis. The maximum specific growth rates of the pure strains cultivation and syntrophic cultivation experiments were compared with the Student's *t*-test for statistically significant difference ($P < 0.05$) and all values presented are the means of independent triplicates (n = 3) ± SD.

RESULTS AND DISCUSSION

Effect of ammonia on hydrogenotrophic methanogens

In general, the methane production of *M. congolense* and *M. thermautotrophicus* decreased with increasing ammonia levels, while the tested ammonia levels had no significant effect ($P > 0.05$) on methane production for *M. bourgensis* and *M. thermophiles* (Fig. 1). The incubation periods of hydrogenotrophic methanogens were the same (about 22–24 days) for all cultivations. Specifically, the growth of *M. congolense* was significantly ($P < 0.05$) affected when ammonia reached 5 g NH_4^+-N L^{-1}. The methane production decreased from 83.3% to 76.2% (compared with theoretical methane production) when ammonia level increased from 3 to 5 g NH_4^+-N L^{-1}. Moreover, the methane production was below detection limits at 7 g NH_4^+-N L^{-1} (Fig. 1c) and thus, *M. congolense* was more sensitive to high ammonia levels compared to the other three hydrogenotrophic methanogens tested.

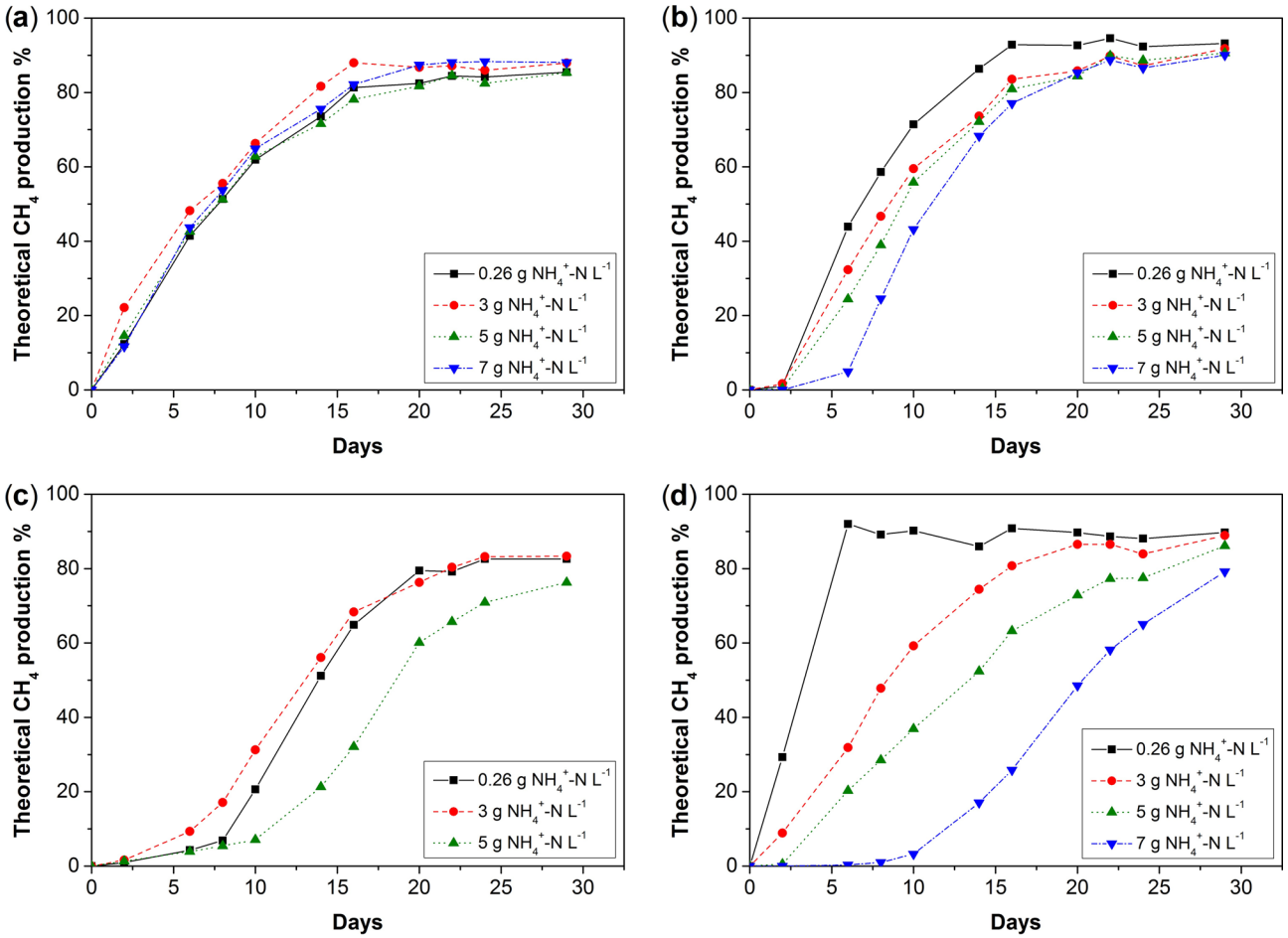

Figure 1. Accumulative methane production under different ammonia levels of (**a**) *M. bourgensis*, (**b**) *M. thermophiles*, (**c**) *M. congolense* and (**d**) *M. thermautotrophicus*.

The methane production for *M. bourgensis* was not significantly ($P > 0.05$) affected by the increased ammonia levels (Fig. 1a). That was in agreement with previous study reporting that *M. bourgensis* was used as a mesophilic fast growing hydrogenotrophic methanogen that can produce methane at high ammonia levels (5 g NH_4^+-N L^{-1}) for bioaugmentation in CSTR reactors (Fotidis *et al.* 2014). Therefore, *M. bourgensis* is robust to high ammonia levels. Similarly, there was no significant decrease ($P > 0.05$) in the methane production of *Methanoculleus thermophilus* (methane production remained above 90% of the theoretical methane production) for all ammonia levels tested (Fig. 1b). The methane production yield of *M. thermautotrophicus* decreased from 89.7% to 79.1%, compared to the theoretical methane production, alongside the increase of ammonia levels from 0.26 to 7 g NH_4^+-N L^{-1} (Fig. 1d). This behaviour was similar to Kato, Kosaka and Watanabe (2008) study, where the growth of *M. thermautotrophicus* suffered inhibition after exposure to 7 g NH_4^+-N L^{-1}. Therefore, *M. thermautotrophicus* was more sensitive to ammonia than *M. bourgensis* and *M. thermophiles*. The incubation time of *M. thermautotrophicus* and *M. congolense* was about 22 days. Specifically, when the ammonia level was increased, the growth rate of *M. thermautotrophicus* decreased, resulting in increase of the growth duration from 6 to more than 20 days (Fig. 1d) demonstrating that the microorganism was inhibited by ammonia.

The inhibitory pressure on the *M. thermautotrophicus* was attributed to the higher free ammonia concentrations (Angelidaki and Ahring 1994) that the thermophilic hydrogenotrophic methanogens were exposed compared to the mesophilic (Table 1). Interestingly, the higher free ammonia concentrations did not seem to affect the growth of *M. bourgensis* (mesophilic) and especially *M. thermophiles* (thermophilic), which was subjected to the same levels of free ammonia as *M. thermautotrophicus*. Therefore, it seems that there are hydrogenotrophic thermophilic methanogens that can tolerate high ammonia and free ammonia concentrations. Furthermore, there are mesophilic hydrogenotrophic methanogens, which are more sensitive to ammonia inhibition compared to thermophilic methanogens. This result opposes to the general belief (Chen, Cheng and Creamer 2008; Fotidis *et al.* 2013) that thermophilic hydrogenotrophic methanogens are more vulnerable to high ammonia loads compared to the mesophilic hydrogenotrophic methanogens.

Effect of ammonia on syntrophic acetate-oxidizing bacteria

The growth of *T. acetatoxydans* and *T. phaeum* suffered a significant ($P < 0.05$) inhibition as the ammonia concentrations were increased (Fig. 2). Nevertheless, *T. acetatoxydans* was able to grow even at 7 g NH_4^+-N L^{-1} although at a much reduced rate (–88.9%) compared to the growth rate at 0.26 g NH_4^+-N L^{-1}. On contrary, the OD_{600} of *T. phaeum* was below detection limits at 5 and 7 g NH_4^+-N L^{-1} (Fig. 2b). It seems that *T. phaeum* was more

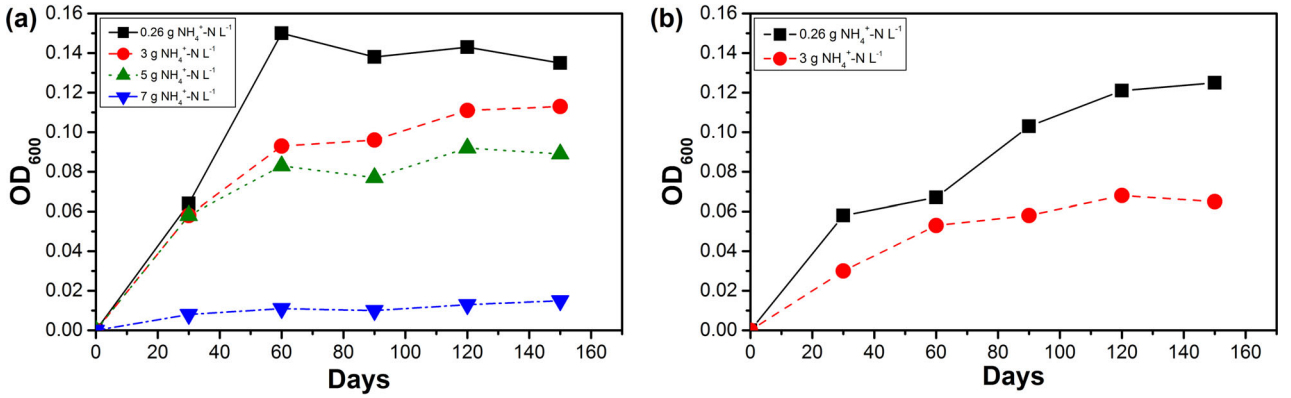

Figure 2. The OD600 under different ammonia levels of (**a**) *T. acetatoxydans* and (**b**) *T. phaeum*.

sensitive to ammonia compared to *T. acetatoxydans* and the reason might be the higher free ammonia concentrations for *T. phaeum* (thermophilic) (Table 1). The results of *T. acetatoxydans* are contradictory to a previous study (Westerholm, Roos and Schnürer 2011) reporting that high ammonia concentration (8.4 g NH_4^+-N L^{-1}) did not have significant impact on the growth of *T. acetatoxydans*. The probable explanation is that an acclimatization process to lower ammonia levels (0.08 g NH_4^+-N L^{-1}) took place during consecutive generations incubated in the medium used by DSMZ to maintain the strain. Additionally, the tolerance to high ammonia levels of *T. phaeum* was in agreement with the results of a previous research (Kato *et al.* 2014). The results clearly showed that both tested SAOB (*T. acetatoxydans* and *T. phaeum*) were more sensitive to high ammonia levels compared to the hydrogenotrophic methanogens tested (except *M. congolense*). This is in contrast to Fotidis, Karakashev and Angelidaki (2013) who reported that methanogens are more sensitive to ammonia inhibition compared to SAOB. Thus, it seems that there are some hydrogenotrophic methanogens equally or more resistant to high ammonia levels compared to some SAOB.

Effect of ammonia on syntrophic cultivation

The methane production of the syntrophically cultivated microorganisms at thermophilic conditions was significantly ($P < 0.05$) decreased (from 56.4% to 0.7% for *M. thermophiles* + *T. phaeum* and from 49.9% to 1.9% for *M. thermautotrophicus* + *T. phaeum* compared to theoretical methane production), when ammonia levels increased from 0.26 to 7 g NH_4^+-N L^{-1} (Fig. 3a and b). Moreover, methane production of the syntrophically cultivated microorganisms at mesophilic conditions was very low (<6% of the theoretical methane production) at 0.26 g NH_4^+-N L^{-1} and was below detection limit at higher ammonia levels. The observed ammonia inhibition to syntrophic cultivation (*M. thermophiles* + *T. phaeum*) was consistent with a previous study (Kato *et al.* 2014). The sensitivity to ammonia of thermophilic syntrophic cultivation was in accordance with the results for *T. phaeum*. The long cultivation times (>150 days for syntrophic cultivation and >120 days for *T. phaeum*) indicated that *T. phaeum* was the most sensitive to ammonia partner, of the syntrophic coculture. The low methane production of syntrophic cultivation microorganisms at mesophilic conditions could be explained by the inability of the *T. acetatoxydans* to oxidize acetate when syntrophically cultivated with *M. bourgensis*, which was consistent with a previous study (Westerholm, Roos and Schnürer 2011). One interesting finding is that there was methane production detected at 5 and 7 g NH_4^+-N L^{-1} (Fig. 3a and b) while the OD_{600} of *T. phaeum* was below detection limits at the same ammonia level (Fig. 2b). It seems that by syntrophically cultivating with hydrogenotrophic methanogens, the overall tolerance of the SAO consortium to ammonia can be improved. A possible explanation for this might be that the syntrophic-cultivated hydrogenotrophic methanogens are reducing hydrogen partial pressure, so the growth of SAOB was stimulated. Thus, the results indicated that hydrogenotrophic methanogens seem to be the crucial factor of the SAO pathway activity, under high ammonia levels.

Maximum growth rate of the pure and syntrophically cultivated strains

In general, the μ_{max} of hydrogenotrophic methanogens (around 0.024 h^{-1}, except *M. congolense*) were significantly higher than SAOB and syntrophic cultivation, which was in agreement of some previous studys [i.e. *M. bryantii* 0.029 h^{-1} (Dubach and Bachofen 1985), *M. bourgensis* 0.022 h^{-1} (Fotidis *et al.* 2014) and *M. thermophiles* + *T. phaeum* 0.004 h^{-1}, (Kato *et al.* 2014)]. Additionally, the μ_{max} of SAOB and syntrophic cultivation was significantly ($P > 0.05$) decreased while the ammonia levels reached 5 g NH_4^+-N L^{-1}. Therefore, it seemed that hydrogenotrophic methanogens were more robust to high ammonia levels compared to SAOB and syntrophic cultivation resulted in enhanced methane production and OD_{600}. Specifically, the μ_{max} of thermophilic syntrophic cultivation (*M. thermophiles* + *T. phaeum* and *M. thermautotrophicus* + *T. phaeum*) decreased by 79.6% and 66.6%, respectively, alongside ammonia from 0.26 to 7 g NH_4^+-N L^{-1}. Under the same condition, the μ_{max} of hydrogenotrophic methanogens (*M. thermophiles* and *M. thermautotrophicus*) only decreased by 26.9% and 16.4%, while for SAOB (*T. phaeum*) there was no growth detected when ammonia reached 5 g NH_4^+-N L^{-1} (Fig. 4a and b). The μ_{max} of mesophilic syntrophic cultivation (*M. bourgensis* + *T. acetatoxydans* and *M. congolense* + *T. acetatoxydans*) was not possible to be calculated at 3 g NH_4^+-N L^{-1} since no methane production was detected (Fig. 4c). At the same time, μ_{max} of *T. acetatoxydans* decreased by 75% when ammonia increased from 3 to 7 g NH_4^+-N L^{-1} (Fig. 4c and d). Conclusively, the profound sensitivity of SAOB to ammonia was the reason for the decrease in μ_{max} of the tested syntrophic coculture. However, it should be noticed that the SAOB tested in this study were only two pure strains (*T. acetatoxydans* and *T. phaeum*) and that there are other pure SAOB strains (*C. ultunense* and *S. schinkii*), which have exhibited high ammonia tolerance under laboratory conditions (Schnürer, Schink and Svensson 1996; Westerholm, Roos and Schnürer 2010, 2011).

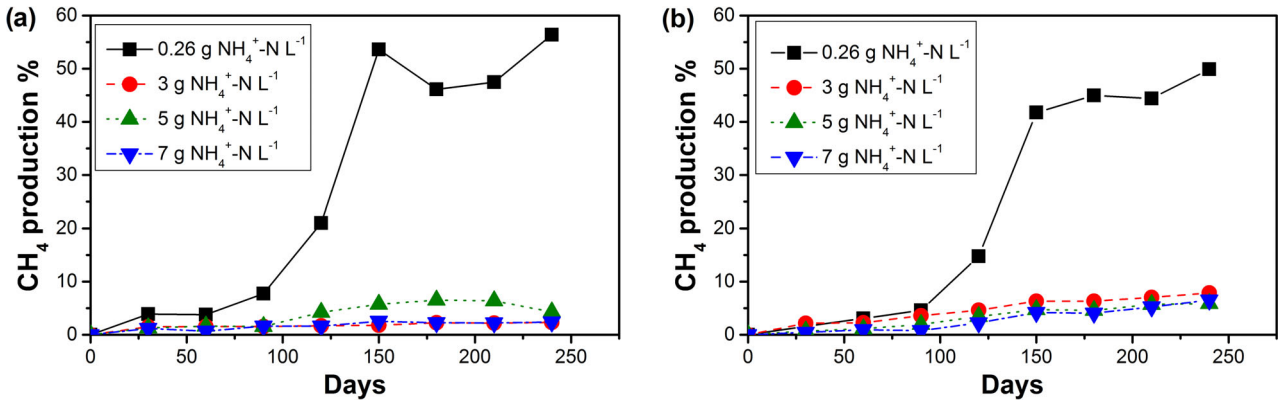

Figure 3. The methane production under different ammonia levels of syntrophically cultivated microbes: (a) *M. thermophiles* + *T. phaeum* and (b) *M. thermautotrophicus* + *T. phaeum*.

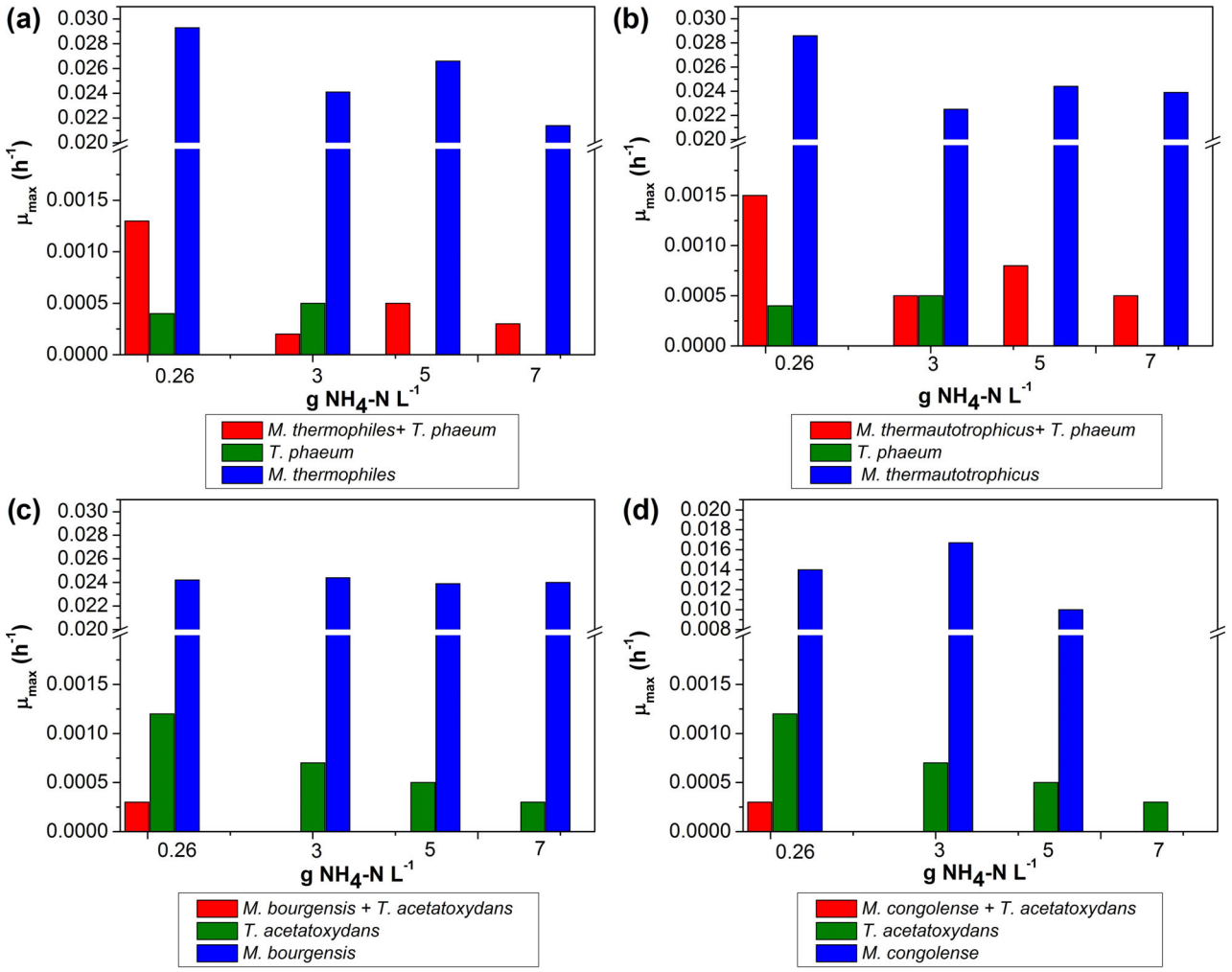

Figure 4. Comparison of the maximum specific growth rates under different ammonia levels of (a) *M. thermophiles* and *T. Phaeum*, (b) *M. thermautotrophicus* and *T. Phaeum*, (c) *M. bourgensis* and *T. acetatoxydans*, (d) *M. congolense* and *T. acetatoxydans*.

Furthermore, many SAOB (*C. ultunense*, *S. schinkii*, *T. acetatoxydans* and *T. phaeum*) in CSTR of large-scale biogas plants have also demonstrated tolerance to ammonia toxicity (Sun *et al.* 2014). However, as we observed in this study SAOB can be more sensitive to ammonia compared to their hydrogenotrophic co-partners.

CONCLUSIONS

The results of the current study indicated that some hydrogenotrophic methanogens were equally or, in some cases, more tolerant to high ammonia concentrations compared to the tested SAOB. Furthermore, it was found that mesophilic

hydrogenotrophic methanogens tested in the current study were more sensitive to ammonia toxicity compared to thermophilic hydrogenotrophic methanogens. These results oppose some investigators who had suggest that, due to higher free ammonia levels, thermophilic hydrogenotrophic methanogens are more vulnerable to high ammonia loads compared to the mesophilic hydrogenotrophic methanogens. These contradictions highlight the fact that the complete knowledge of ammonia inhibition effect on SAOB and hydrogenotrophic methanogens is still lacking. Nevertheless, the growth of SAOB was stimulated under high ammonia levels when cultivated syntrophically with hydrogenotrophic methanogens. Thus, it seems that hydrogenotrophic methanogens are the key players in the SAO pathway under high ammonia concentrations. Overall, this study shown that it is difficult to make generalizations in respect to ammonia inhibition effect on anaerobic digestion microbes.

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Conflict of interest. None declared.

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